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Im Auftrag

For the President of the European Patent Office

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EUKARYOTIC CELL-BASED GENE INTERACTION CLONING

The present invention relates to a method for screening compounds for their ability to bind a receptor and/or the screening of compounds that antagonise the binding of a ligand to a receptor.

Receptors are defined as proteinaceous macromolecules often located on cell membranes that perform a signal transducing function. Many receptors are located on the outer cell membrane. Several receptors possess three domains, the extracellular domain, the transmembrane domain and the cytoplasmic domain. The extracellular domain is capable of specifically binding to a compound, normally called "ligand". Signal transduction appears to occur in a variety of ways upon ligand binding, such as for example by a conformational change in the structure of the receptor, by clustering of two identical or related receptor-type molecules.

Many receptors have been identified and the scientific literature has variously divided them into groups, superfamilies, families and/or classes of receptors based on common features such as tissue distribution of the receptors, nucleic acid or amino acid homology of the receptors, mechanisms of signalling by the receptors or the type of ligand that binds to the receptors. A uniform system of classifying or grouping receptors, however, has not been used in the literature.

It is well established that polypeptide hormones elicit their biological effect by binding to receptors expressed on the surface of responsive cells. At least four families of polypeptide hormone receptors can be defined on the basis of similarity in primary sequence, predicted secondary and tertiary structure and biochemical function. These are the haemopoietin/interferon receptor family, the receptor kinase family, the tumour necrosis factor (TNF) / nerve growth factor (NGF) family and the family of G-protein coupled receptors. The haemopoietin/interferon family receptors have no intrinsic enzymatic activity; they can be recognised on the base of their "cytokine receptor homology"

(CRH) region in their extracellular domains. This CRH region contains two conserved cystein bridges and a tryptophan - serine - X - tryptophan - serine motif. The defining features of members of the TNF-NGF receptor family are located in the extracellular domain and centre on a domain that contains 6 cysteine residues. The receptor kinase family is characterised by a conserved catalytic kinase domain in the cytoplasmic part of the receptor; the family is subdivided in tyrosine kinase and serine/threonine kinase receptors, on the base of their substrate specificity. While receptors in the haemopoietin, TNF/NGF and kinase families contain a single transmembrane domain, G-protein coupled receptors traverse the membrane several times. With the exception of the G-protein coupled receptors, cytokine driven multimerization of the receptor subunits appears to be the initial event in signal transduction. While homo- or heterodimerization and trimerization are central to the function of haemopoietin / interferon receptors and TNF / NGF receptors, respectively, homodimerization appears a preferred way of receptor kinase action.

A special case is that of the receptor-like protein tyrosine phosphatases. All members possess an intracellular part containing one or two homologous protein tyrosine phosphatase domains, a single membrane spanning region and variable extracellular segments with potential ligand binding capacity.

As described above, cytokine-driven interaction between receptor subunits appears to be the initial event for haemopoietin / interferon receptors. The recognition of the ligand starts with one receptor subunit; this subunit is often called a-subunit in case of heteromeric receptors. After this initial event, there is an association of one or more additional receptor molecules, which is essential for the initiation of the signal transduction and, as an additional effect can lead to an increase in affinity of the ligand binding. Receptor clustering leads to activation of the kinase function. The haemopoietin / interferon receptors which, contrary to the tyrosine kinase receptors, do not have an intrinsic kinase activity, are using the help of the associated "Janus kinases" (JAKs) to phosphorylate the tyrosine residues. Subsequent targets for the JAKs include the JAK molecules themselves, the cytoplasmic part of the receptor and the "Signal Transducers and Activators of Transcription"

proteins (STAT). This pathway is called the "JAK / STAT pathway". Additional pathways, such as the Ras - Raf - mitogen activated protein kinase pathway may also be activated.

Examples of the haemopoietin / interferon receptors are, amongst others, the interleukin-5 (IL-5) receptor, the erythropoietin receptor and the interferon receptor family.

The IL-5 receptor is a heteromer consisting of two subunits. The IL-5 receptor a-chain is ligand specific and has a low to intermediate binding affinity. Association with the IL-5 receptor b chain, that is common with other receptor complexes such as IL-3, results in a high affinity binding complex. Both receptor subunits are required for signalling. Furthermore, signalling requires the cytoplasmic tails of both receptor subunits.

Interferons are classified into two classes. Type one interferons consist of the IFNa group, IFNb, IFNw and the bovine embryonic form, IFNt. IFNg belongs to the second group. The receptor complex of the type I interferons consists of an IFNaRI subunit and an IFNaRII subunit. The latter receptor chain exists in three isoforms, resulting from alternative splicing:IFNaRII-1 and IFNaRII-2 are membrane associated but differ in length of the cytoplasmic domain, whereas IFNaRII-3 is a soluble form.

A lot of information about the signal transduction process of these receptors has been obtained by genetic complementation studies, using the 2ftGH cell line (Pellegrini *et al.,* 1989; Darnell *et al.,* 1994) and the 6-16 promoter (Porter *et al.,* 1988). The human 2ftGH cell line is hypoxanthine-guanine phosphoribosyl transferase (HGPRT) deficient, but is containing the xanthine guanine phosphoribosyl transferase (*gpt*) gene of *E. coli*, under the control of the type I IFN inducible 6-16 promoter. In cell lines with a functional IFNa or b receptor (IFNaR), the 6-16 promoter becomes induced and the *gpt* gene is transcribed, when IFNa or b is added to the medium. The enzyme produced, xanthine guanine phosphoribosyl transferase (XGPRT) is able to complement the HGPRT deficiency. This allows a positive or a negative selection. Positive selection (growth of XGPRT producing cells) is carried out on hypoxanthine

aminopterine thymidine (HAT) medium, negative selection (dead of XGPRT producing cells) is carried out on DMEM medium with 6-thioguanine (6-TG).

The study of receptor-ligand interactions has revealed a great deal of information about how cells respond to external stimuli. This knowledge has led to the development of several therapeutically important compounds. However, many molecules that control cell growth and development are not yet discovered and there exist so called "orphan receptors", of which the ligand(s) are unknown.

Several methods have been proposed to screen for ligands of orphan receptors. Kinoshita *et al.*(1995) developed a functional screen in yeast to identify ligands for receptor tyrosine kinases. This method is hampered by the need to have functional expression of the receptor genes in the yeast host. US 5597693 describes a screening method in mammalian cells that is, however, limited to intracellular receptors of the steroid/thyroid superfamily and can not be used for cytokine receptors. WO 95/21930 describes a screening method for cytokine receptors. In this method, ligands are screened after random mutagenesis of a cell line. Only those ligands can be detected of which the expression can be activated by mutagenesis in the cell type used. Moreover, the isolation of the ligand encoding genes is rather complicated. This is a severe restriction for the usefulness of the screening method. In WO 96/02643, a method is described to screen for ligands of the Denervated Muscle Kinase (DMK) receptor and chimeric variants thereof. However, the applicability of this method is rather limited and there is no direct, rapid way provided to isolate the genetic material encoding the ligand.

It is the aim of the present invention to provide an easy and powerful screening method in eukaryotic cells, such as insect cells, plant cells or mammalian cells, with the exclusion of yeast cells, for ligands of orphan receptors, preferentially of the multimerizing receptor type, for unknown ligands of known receptors, preferentially multimerizing receptors and for the genes encoding these ligands. Hereto, chimeric receptors are constructed, comprising an extracellular domain derived from one protein, preferentially the extracellular domain of a receptor, and a cytoplasmic part derived from

another protein which should be a receptor; at least one chimeric receptor is expressed in a eukaryotic host cell which is not a yeast cell. The same eukaryotic host cell comprises a recombinant gene, encoding for a compound of which the expression creates an autocrinic loop, and a reporter system that is activated upon the creation of said autocrinic loop. Preferentially, the compound of which the expression creates an autocrinic loop is a ligand for the chimeric receptor. When this autocrinic loop is closed, the reporter system is switched on, preferentially by the use of a promoter that can be activated by binding of said ligand to said chimeric receptor.

All three elements (chimeric receptor, recombinant gene, reporter system) can be either stably transformed into the eukaryotic cell, or transiently expressed.

Moreover, it is clear, for people skilled in the art, that the autocrinic loop can be more complex, and may consist of more than one loop. As a non limiting example, the recombinant gene may express the ligand of a first (chimeric or non-chimeric) receptor that activates a second gene which upon activation expresses the ligand of a second receptor, of which the ligand binding results in the induction of the reporter system. It is even not essential that the first and the second receptor are situated within the same cell: it is clear, for people skilled in the art, that one can work with two cell populations, the first one carrying a recombinant gene, expressing a ligand for a receptor for the second cell, which upon binding of the ligand starts to produce the ligand of the chimeric receptor, situated on the first cell. Binding of the latter ligand to the chimeric receptor then results in the expression of the reporter system.

In a first embodiment, the *gpt* selection system can be applied to the screening and/or selection of orphan receptors. Hereto, the extracellular domain of the receptor that is studied is fused to the intracellular domain(s) of IFNaR. The receptor studied may be an orphan receptor or a known receptor. The use of the IFN receptor cytoplasmic tails guarantees correct signalling required for reporter activation, independent of the function (which may be unknown) of the receptor studied, as long as it functions by clustering. The ligand is supplied by the creation of an autocrinic loop: cells are transfected

by a DNA expression library, where genes, encoding for possible ligands for the orphan receptor, are placed preferentially after a strong, constitutive promoter. It is known, however, to people skilled in the art that other promoters can be used, such as inducible promoters and even an IFN inducible promoter. The production of the cognate ligand induces the transcription of the *gpt* gene, enabling a positive selection in HAT medium. Alternatively, candidate ligands can be added to the medium; survival of the cells in the HAT medium will only be detected when a ligand can bind the orphan receptor.

In a second embodiment, secreted alkaline phosphatase (SEAP) may be used as reporter system. Cells expressing the reporter system can be identified by measuring the SEAP activity using CSPD (disodium 3-(4-methoxyspirol-1,2-dioxetane-3,2'-(5'-chloro)trichloro {3.3.1.1(3,7)}decan-4-yl)phenyl phosphate) as luminogenic substrate.

The invention is not limited to the use of the cytoplasmic tails of the interferon receptor and the *gpt* selection system, but other receptor systems and/or other inducible promoters and/or other reporter systems and/or other cell lines, known to people skilled in the art may be used. As a non limitative example, PC12 cells (Green *et al.*, 1976), with a chimeric receptor based on the leptin receptor (Tartaglia *et al.*, 1995) and the Pancreatitis associated protein I as inducible promoter may be used. The reporter system may be based upon the detection of the gene product of an inducible gene, as is the case for Green Fluorescent Protein (GFP) as a non limiting example, or may be based on modification of a protein already present in the cell (proteolytic cleavage, phosphorylation, complex formation...) such as the systems described by Mitra *et al.* (1995), Miyawaki *et al.* (1997) and Romoser *et al.* (1997). Moreover, optimal reporter activation may require a co-stimulus, as is the case for the leptin-forskolin system.

A further aspect of the invention is the screening of compounds that are antagonists of the ligand-receptor binding. Due to the fact that can be screened for the toxicity of *gpt* expression in D-MEM + 6-TG medium, it is possible to set up an antagonistic screening system for compounds that

inhibit and/or compete with the binding of the ligand to the chimeric receptor. This can be realized by using the autocrinic loop and adding possible inhibitors to the medium, but it is clear for people skilled in the art that, alternatively, the cell can be transformed with genes encoding candidate inhibitors. Expression of an inhibitor would create an anti-autocrinic loop. In this case, the ligand is produced either by an autocrinic loop, or added to the medium,or the receptor may be mutated and/or genetically modified to a form that constitutively initiating the signalling pathway. Such a screening may be useful in the identification of compounds with potential pharmaceutical applications.

A further aspect of the invention is the screening of compounds in the signalling pathway: a host cell, carrying the chimeric receptor and the gene for its ligand, placed after a promoter, in principle inducible by the chimeric receptor, but where said host cell is missing one or more compounds of the signalling pathway, can be transfected by an expression library in order to complement the signalling pathway. Complemented cells will be detected by the activation of the reporter system. This method could be extremely useful in case a receptor with unknown signalling pathway is placed in the autocrinic loop, before or after the loop that is activating the chimeric receptor.

Still another aspect of the invention is the screening of compounds that are involved in the secretory pathway: as the ligand for the chimeric receptor needs to be secreted in order to activate the receptor, both compounds that block the secretion, or compounds that can complement a mutation in the secretory pathway can be screened.

Definitions

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

multimerizing receptor: every receptor of which the binding of the ligand results in the multimerization of the receptor components, and/or every

protein that can be identified by the people skilled in the art as such a receptor on the base of its amino acid sequence and/or protein structure.

Multimerization can be homo- or heterodimerization, homo- or heterotrimerization, ..., up to complex formation of multiple proteins.

Orphan receptor: every receptor, preferentially a multimerizing receptor, or protein with known receptor components of which no ligand is known that is binding to this receptor and, as a consequence, initiating the signalling pathway.

Ligand: every compound that can bind to a receptor, preferentially a multimerizing receptor and that is initiating the signalling pathway by its binding to said receptor.

Unknown ligand: every compound that can bind to a receptor, preferentially a multimerizing receptor and that is initiating the signalling pathway by its binding to said receptor, but for which this binding has not yet been demonstrated.

Compound: means any chemical or biological compound, including simple or complex inorganic or organic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof.

Extracellular domain: means the extracellular domain of a receptor and/or orphan receptor, or a functional fragment thereof characterised by the fact that it still can bind a known and/or unknown ligand, or a fragment thereof fused to other amino acid sequences, characterised by the fact that it still can bind a known and/or unknown ligand, or a fragment from a non-receptor protein that can bind a known and/or unknown ligand.

Bind(ing) means any interaction, be it direct (direct interaction of the compound with the extracellular domain) or indirect (interaction of a compound with one or more identical and/or non-identical compounds resulting in a complex of which one or more compounds can interact with the extracellular domain), that result in initiating the signalling pathway of the chimeric receptor

Cytoplasmic domain: means the cytoplasmic part of a receptor, or a functional fragment thereof, or a fragment thereof fused to other amino acid sequences, capable of initiating the signalling pathway of said receptor and of inducing a reporter system.

Chimeric receptor: functional receptor comprising an extracellular domain and the cytoplasmic domain of a receptor.

Reporter system: every compound of which the synthesis and/or modification and/or complex formation can be detected and/or be used in a screening and/or selection system. The reporter system can be, as a non limiting example, a gene product encoding an enzymatic activity, a coloured compound, a surface compound or a fluorescent compound.

Autocrinic loop: every succession of events by which a cell, carrying a receptor allows the synthesis of the ligand and/or unknown ligand for said receptor.

Anti-autocrinic loop: every succession of events by which a cell, carrying a receptor allows the synthesis of a compound that inhibits the binding of a ligand and/or unknown ligand to said receptor

Signalling pathway: means every succession of events after the binding of a ligand and/or unknown ligand to an extracellular domain of a natural occurring

or chimeric receptor whereby said binding can result in the induction and/or repression of a set of genes.

Selection: means isolation and/or identification of cells in which the reporter system is activated or isolation and/or identification of cells in which the reporter system is not activated.

Examples

I. CONSTRUCTION OF THE CHIMERIC RECEPTORS

I.1. Construction of IL-5R/IFNaR chimeric receptors

I.1.1 Construction in the pcDNA3 vector

All polymerase chain reactions (PCR) were performed using the Expand High Fidelity PCR system kit (Boehringer Mannheim). This kit is supplied with an enzyme mix containing thermostable Taq DNA and Pwo DNA polymerases (Barnes et al, 1994). The IL-5Rα extracellular domain sequence (amino acids 1-341, not including the last Trp342 residue) was amplified by PCR using the forward primer MBU-O-37 that contains a Kpn I site and the reverse primer MBU-O-38 (table 1). The sequence encoding the βc extracellular domain (amino acids 1-438, not including the last Val439 residue) was PCR amplified using the forward primer MBU-O-39 which also contains a KpnI site and the reverse primer MBU-O-40. A forward primer MBU-O-41 was used with a reverse primer MBU-O-42, which contains an XhoI site, to amplify the sequence that codes for the IFNaR1 transmembrane (TM) and intracellular (IC) domain (amino acids 436-557, including the last residue of the extracellular domain, Lys436). The forward primer MBU-O-43 was used to amplify the sequence encoding the IFNaR2-1 transmembrane and intracellular domains (amino acids 243-331, including the last residue of the extracellular domain, Lys243) and the IFNaR2-2 TM and IC domains (amino acids 243-515, including the last residue of the extracellular domain, Lys243), respectively in combination with the reverse primers MBU-O-44 and MBU-O-45, containing an XhoI site. After gel purification, and phosphorylation, six combinations of PCR fragments encoding for the EC on the one hand and for

the TM + IC domains on the other hand, were ligated and subsequently used as input DNA in a second PCR reaction:

1) IL-5Rα extracellular domain fragment + IFNaR1 intracellular domain fragment, using MBU-O-37 and MBU-O-42 as forward and reverse primers, respectively.

2) IL-5Rα extracellular domain fragment + IFNaR2-1 intracellular domain fragment, using MBU-O-37 and MBU-O-44 as forward and reverse primers, respectively.

3) IL-5Rα extracellular domain fragment + IFNaR2-2 intracellular domain fragment, using MBU-O-37 and MBU-O-45 as forward and reverse primers, respectively.

4) βc extracellular domain fragment + IFNaR1 intracellular domain fragment, using MBU-O-39 and MBU-O-42 as forward and reverse primers, respectively.

5) βc extracellular domain fragment + IFNaR2-1 intracellular domain fragment, using MBU-O-39 and MBU-O-44 as forward and reverse primers, respectively.

6) βc extracellular domain fragment + IFNaR2-2 intracellular domain fragment, using MBU-O-39 and MBU-O-45 as forward and reverse primers, respectively.

The resultant blunt PCR fragments, coding for the hybrid receptors, were isolated by agarose gel electrophoresis, digested with KpnI - XhoI and ligated into the KpnI-XhoI opened pcDNA3 vector (Invitrogen).
The constructs were checked by DNA sequence analysis and named as follows: pcDNA3-IL-5Rα/IFNaR1, pcDNA3-IL-5Rα/IFNaR2-1, pcDNA3-IL-5Rα /IFNaR2-2, pcDNA3-βc/IFNaR1, pcDNA3-βc/IFNaR2-1 and pcDNA3-βc/IFNaR2-2.

Table 1 : oligonucleotides used for construction of chimeric receptors and IL-5 expression vectors.

Number	Specification	forward/reverse	Sequence (5'-3')
MBU-O-37	hIL5Ralpha nt.251-268	Forward	GCTGGTACCATGATCATCGTGGCGCATG
MBU-O-38	hIL5Ralpha nt.1272-1252	Reverse	CTCTCTCAAGGGCTTGTGTTC
MBU-O-39	hbetac nt.29-49	Forward	GCTGGTACCATGGTGCTGGCCCAGGGGCTG
MBU-O-40	hbetac nt.1343-1322	Reverse	CGACTCGGTGTCCCAGGAGCG
MBU-O-41	hIFNaR1 nt.1384-1403	Forward	AAAATTTGGCTTATAGTTGG
MBU-O-42	hIFNaR1 nt.1743-1764	Reverse	CGTCTCGAGGTTCATTTCTGGTCATACAAAG
MBU-O-43	hIFNaR2-1 nt.952-971	Forward	AAAATAGGAGGAATAATTAC
MBU-O-44	hIFNaR2-1 nt.491-468	Reverse	CGTCTCGAGACATAATAAAACTTAATCACTGGG
MBU-O-45	hIFNaR2-2 nt.1275-1257	Reverse	CGTCTCGAGATAGTTTTGGAGTCATCTC
MBU-O-278	PacI mutagenesis in IL-5Ralpha/IFNaR2-2	forward	CACAAGCCCTTGAGAGAGTTAATTAAAATAGGAGGAATAATTACTG
MBU-O-279	PacI mutagenesis in IL-5Ralpha/IFNaR2-2	reverse	CAGTAATTATTCCTCCTATTTTAATTAACTCTCTCAAGGGCTTGTG
MBU-O-280	PacI mutagenenesis in beta/IFNaR1	forward	CCTGGGACACCGAGTCGTTAATTAAAATTTGGCTTATAGTTGG
MBU-O-281	PacI mutagenenesis in beta/IFNaR1	reverse	CCAACTATAAGCCAAATTTTAATTAACGACTCGGTGTCCCAGG
MBU-O-167	hEPO-R primer nt. 105	Forward	CGGGGTACCATGGACCACCTCGGGGCGTCC
MBU-O-308	hEPO-R primer nt. 872	Reverse	CCCTTAATTAAGTCCAGGTCGCTAGGCGTCAG
MBU-O-187	Linker for pMET7-MCS	Sense	TCGACTCAGATCTTCGATATCTCGGTAACCTCACCGGTTCCTCGAGTCT
MBU-O-188	Linker for pMET7-MCS	antisense	CTAGAGACTCGAGGAACCGGTGAGGTTACCGAGATATCGAAGATCTGAG

I.1.2. Construction in the pSV-SPORT vector and insertion of a PacI site

As an alternative, we also tested the chimeric receptors in the pSV-SPORT expression vector (Life Sciences). This vector contains an SV40 early promoter which is normally weaker as compared to the CMV promoter of the pcDNA3 plasmid.

The genes for the chimeric receptors in pcDNA3-IL-5Rα/IFNaR2-2 and pcDNA3-βc/IFNaR1 were isolated by Asp718 and XhoI digestion and agarose gelelectrophoresis, followed by insertion in the Asp718-SalI opened pSV-SPORT vector. The resulting constructs were verified by sequence analysis and named pSV-SPORT-IL-5Rα/IFNaR2-2 and pSV-SPORT-βc/IFNaR1.

In addition, we inserted a unique PacI restriction site immediately preceding the last amino acid codon of each extracellular domain (Trp341 and Val438 for IL-5Rα and βc, respectively). This enabled us to quickly exchange the IL-5R extracellular domains with the extracellular domains of other receptors. Insertion mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene), using the oligonucleotides MBU-O-278 (sense) and MBU-O-279 (antisense) for IL-5Rα/IFNaR2-2 and MBU-O-280 (sense) and MBU-0-281 (antisense) for βc/IFNaR1 (table1). As a result, two amino acids (Leu-Ile) were inserted in the membrane-proximal region of the extracellular domain, which did not interfere with receptor functionality. The resulting plasmids were named pSV-SPORT-IL5RaP/IFNaR2-2 and pSV-SPORT-bcP/IFNaR1

I.2. Construction of EPO-R/IFNaR chimeric receptors

RNA was prepared from $5x10^6$ TF-1 cells according to the procedure of the RNeasy kit (Qiagen), and dissolved in 50µl water from which 10µl was used for RT-PCR. To these, 2 µl (2µg) of oligodT (12-18 mer; Pharmacia) was added and incubated at 70°C for 10 min. After chilling on ice for 1 min., cDNA was prepared by adding 4µl of RT buffer (10x; Life Sciences), 1 µl dNTP's (20 mM; Pharmacia), 2µl DTT (0.1M) and 1 µl of MMLV reverse

transcriptase (200U; superscript; Life Sciences) so that the total volume was 20 µl. Incubations were successively at RT for 10 min., 42°C for 50 min., 90°C for 5 min. and 0°C for 10 min.. Following this, 0.5 µl RnaseH (2 U; Life Sciences) was added and the mixture was incubated at 37°C for 20 min., followed by chilling on ice. For PCR amplification of the DNA, 5 µl of this mixture was diluted in 17 µl water followed by addition of 1 µl dNTP's (20 mM), 5µl Pfu buffer (10x; Stratagene), and 10 µl (100 ng) of forward and reverse primer for EPO-R (MBU-0-167 and MBU-0-308, respectively, see table 1). The PCR was started at 94°C for 2 min. during which 2 µl Pfu enzyme (5 U; Stratagene) was added (hot start) and followed by 40 cycles with denaturation at 92°C (1 min.), hybridization between 55 till 59°C (1 min.; with an increasing temperature gradient over 4°C during the 40 cycles) and polymerization at 72°C (3 min.; with a 0.05 min. elongation time during every cycle, but only in the last 25 cycles). To finalise, the reaction was hold on 72°C for 12 min. and chilled to 4°C. A band of correct size was isolated from an agarose gel and the DNA was digested with PacI and KpnI and inserted into the PacI-KpnI opened pSV-SPORT-IL-5RαP/IFNaR2-2 or pSV-SPORT-β cP/IFNaR1 vectors. The resultant vectors were named pSV-SPORT-EPO-R/IFNaR2-2 and EPO-R/IFNaR1, respectively.

FUNCTIONALITY OF THE CHIMERIC RECEPTORS

II.1. IL-5 can activate the 6-16 promoter via IL-5R/IFNaR chimeric receptors.

II.1.1. Activation of 6-16 *gpt* allows selection of stabile colonies.

The following nine combinations of plasmids were transfected in 2ftGH cells:

1. pcDNA3-IL-5Rα/IFNaR1 + pcDNA3-βc/IFNaR1
2. pcDNA3-IL-5Rα/IFNaR1 + pcDNA-βc/IFNaR2-1
3. pcDNA3-IL-5Rα/IFNaR1 + pcDNA-βc/IFNaR2-2
4. pcDNA3-IL-5Rα/IFNaR2-1 + pcDNA-βc/IFNaR1
5. pcDNA3-IL-5Rα/IFNaR2-1 + pcDNA-βc/IFNaR2-1
6. pcDNA3-IL-5Rα/IFNaR2-1 + pcDNA-βc/IFNaR2-2
7. pcDNA3-IL-5Rα/IFNaR2-2 + pcDNA-βc/IFNaR1

8. pcDNA3-IL-5Rα/IFNaR2-2 + pcDNA-βc/IFNaR2-1
9. pcDNA3-IL-5Rα/IFNaR2-2 + pcDNA-βc/IFNaR2-2

pcDNA3 alone was used for mock transfection.

Transfection was according to the calcium phosphate method (Graham and van der Eb (1973), Virology 52, 456) . For each plasmid, 10 µg DNA was used (20 µg of pcDNA3 for mock transfection). The precipitate was made up in 1 ml and left on the cells overnight ($5x10^5$ cells/transfection/petridish). The dishes were then washed twice with Dulbecco's PBS (Life Sciences) and cells were left in DMEM (Life Sciences). 48 hours later, DMEM medium + G418 (Calbiochem; 400 µg/ml) was added. 3 days later, cells from every transfection were trypsinized with 5 ml 0.05% trypsine / 0.02% EDTA solution (Life technologies) and seeded in three wells of a 6-well microtiterplate. The day after, 1) HAT medium (Life Sciences) alone + G418, 2) HAT medium + G418 + 500 U/ml IFNα2b (PeproTech, Inc) or 3) HAT medium + G418 + 1 ng/ml IL-5 (produced in Sf9 cells using published methodologies) was added. 6 days later, small colonies appeared only in the IL-5Rα/IFNaR1 + β c/IFNaR2-2 and IL-5Rα/IFNaR2-2 + βc/IFNaR1 transfections, when the cells were incubated with HAT + G418 + IL-5, indicating that these IL-5R/IFNaR chimeric receptors were functional in that they transmitted the signal to activate the 6-16 promoter. In none of the transfections, growth in HAT medium alone resulted in clear colony formation, while in all transfections, incubation with 500 U/ml IFNα resulted in 50-100 colonies (see table 2).

Table 2

	HAT	HAT + IL-5	HAT + IFNα
IL-5Rα/IFNaR1 + βc/IFNaR1	-	-	+/- 75
IL-5Rα/IFNaR1 + βc/IFNaR2-1	-	-	+/- 50
IL-5Rα/IFNaR1 + βc/IFNaR2-2	-	3	+/- 50
IL-5Rα/IFNaR2-1 + βc/IFNaR1	-	-	+/- 75
IL-5Rα/IFNaR2-1 + βc/IFNaR2-1	-	-	+/- 100
IL-5Rα/IFNaR2-1 + βc/IFNaR2-2	-	-	+/- 100
IL-5Rα/IFNaR2-2 + βc/IFNaR1	-	13	+/- 100
IL-5Rα/IFNaR2-2 + βc/IFNaR2-1	-	-	+/- 100
IL-5Rα/IFNaR2-2 + βc/IFNaR2-2	-	-	+/- 50
mock	-	-	+/- 100

The experiment was repeated twice, with slight modifications in the procedures according to time of adding supplements, changing media and length of incubation times, but similar ratios were obtained (data not shown).

To isolate single clones, cells stabile transfected with the combinations pcDNA3-IL-5Rα/IFNaR1 + pcDNA3-βc/IFNaR2-2 or pcDNA3-IL-5Rα/IFNaR2-2 + pcDNA3-βc/IFNaR1, were further cultivated for two days in DMEM medium + HT supplement, allowing cells to switch back to normal DMEM medium. Single cells were isolated by limited dilution in a 96-well microtiterplate and resulting colonies were further grown in DMEM for two weeks for depletion of *gpt*, and stored. 6 colonies of each transfection were further investigated on their IL-5 responsiveness by re-analysing their growth behaviour in HAT medium alone, HAT medium + IL-5, or DMEM medium.
Using an inverted microscope, cell survival was visually followed during a two week period and selection of an optimal clone was based on 1) rapid growth in HAT + IL-5 which correlates with rapid growth in DMEM, and 2) pronounced cell death in HAT alone. One clone was selected for each combination: IL-5Rα/IFNaR1 + βc/IFNaR2-2 clone B and IL-5Rα/IFNaR2-2 + βc/IFNaR1 clone C.
2ftGH cells that were stabile transfected with the pSV-SPORT IL-5Rα /IFNaR2-2 + pSV-SPORT βc/IFNaR1 vectors were isolated essentially the same way with the exception that selection in G418 medium was omitted. For each plasmid, 10 μg DNA was used (20 μg of pSV-SPORT for mock transfection). The precipitate was made up in 1 ml and left on the cells overnight ($5x10^5$ cells/transfection/petridish). The dishes were then washed twice with Dulbecco'sPBS and cells were left in DMEM. 24 hours later, cells from every transfection were trypsinized with 5 ml 0.05% trypsine / 0.02% EDTA solution (Life technologies) and seeded in three wells of a 6-well microtiterplate. The day after, 500 U/ml IFNα or + 1 ng/ml IL-5 was added or cells were left unstimulated and 24 hours later the medium was removed and replaced by HAT medium with the same stimuli or without stimulus. About 14 days later, small colonies appeared, when the cells were incubated with HAT

+ IL-5. In none of the transfections, growth in HAT medium alone resulted in clear colony formation, while in all transfections, incubation with 500 U/ml IFN α resulted in a confluent monolayer. Isolation of single colonies was accomplished essentially the same way as described above. A clone with the best response to IL-5 in HAT medium and in medium supplemented with 6-TG was called CloneE.

The cells developed at this stage could already serve as an assay system for the evaluation of exogeneously added ligands.

II.1.2. Construction of p6-16SEAP and development of the 2ftGH-6-16SEAP stabile cell line.

Although formation of stable colonies is a reliable and reproducible assay to investigate chimeric receptor activation, this method suffers from the disadvantage that it is very time-consuming and cannot be used for quantification of receptor functionality. We therefore constructed a plasmid wherein the 6-16 promoter was cloned into the pSEAP vector (Tropix), upstream the reporter gene coding for secreted alkaline phosphatase (SEAP). The SEAP reporter gene was selected since the IFN signaling pathway was found to interfere with luciferase and β-galactosidase activities (data not shown). A HindIII fragment that contained the entire 6-16 promoter was isolated from the plasmid 6-16luci (gift from Sandra Pellegrini, Institut Pasteur, Paris) and inserted in the HindIII-opened pSEAP vector so that the 6-16 promoter was in front of the SEAP gene. The resultant plasmid was named p6-16SEAP.

Stabile 6-16SEAP transfected 2ftGH cell lines were obtained by co-transfection of 20 µg p6-16SEAP with 2 µg pBSpac/deltap (obtained from the Belgian Coordinated Collections of Microorganisms, BCCM)in the 2ftGH cells. The latter plasmid contained a gene for puromycin resistence under control of the constitutive SV40 early promoter. Selection on puromycin was on the basis of methods descibed in the art. We choose 3 µg puromycin/ml as an optimal concentration for selection of puromycin-resistant 2ftGH cells. To isolate single colonies, the double transfected cells were trypsinised and

colonies were grown by limited dilution in puromycin-containing medium. Selection of the two best responding clones was based on SEAP production after IFNα and β stimulation in comparison when no stimulus was added. These clones were named 2ftGH-6-16SEAPclone2 and 2ftGH-6-16SEAPclone5.

II.1.3. Activation of the 6-16SEAP reporter by IL-5 in transient transfection assays

10 µg of pSV-SPORT-IL-5Rα/IFNaR2-2 and 10µg of pSV-SPORT-βc/IFNaR1 were co-transfected in 2ftGH cells, together with 10µg of the plasmid p6-16SEAP. Transfection was according to the Ca-phosphate procedure. The precipitate was made up in 1 ml and equally dispersed over four wells in a 6-well microtiterplate (165 µl/10^5 cells/well) and left on the cells overnight. Cells were washed twice the next day (2 x with Dulbecco's PBS)and further grown in DMEM medium for 24 hours. The day after, IFNβ (500U/ml; IFNb1a, gift from P. Hochman, Biogen, Cambridge) or IL-5 (0, 1 and 2 ng/ml) was added and the cells were left for another 24 hours. Finally, samples of medium from each well were taken to assay for SEAP activity with the Phospha-Light kit (Tropix), using CSPD as a luminogenic substrate and light production was measured in a Topcount luminometer (Canberra-Packard). Comparison with untreated cells shows a 2.5-fold increase in SEAP activity when the cells were treated with IFNβ as compared to untreated cells, and a 5-to 6-fold increase when cells were stimulated with 1 or 2 ng/ml IL-5, respectively (figure 1).

II.2. Erythropoietin can activate the 6-16 promoter via EPO-R/IFNaR chimeric receptors.

II.2.1. Activation of 6-16 SEAP in transient transfection assays

20 µg of pSV-SPORT-EPO-R/IFNaR2-2 alone, 20 µg of pSV-SPORT-EPO-R/IFNaR1 alone, 10µg of pSV-SPORT-EPO-R/IFNaR1 + 10 µg of pSV-SPORT-EPO-R/IFNaR2-2 or 10 µg of pUC18 alone (mock; Pharmacia) were transfected in 2ftGH-6-16SEAPclone2 cells. The precipitate was made up in 1 ml and left on the cells for six hours (5x10^5 cells/transfection/petridish). The

dishes were then washed twice with Dulbecco'sPBS and cells were further grown in DMEM. After 24 hours, cells from every transfection were trypsinized with 5 ml 0.05% trypsine / 0.02% EDTA solution (Life technologies) and seeded in three wells of a 6-well microtiterplate. The next day, IFNα (500U/ml) or erythropoietin (EPO, 0.5 U/ml, R&D systems) was added and the cells were left for another 24 hours. Finally, samples of medium from each well were taken to assay for SEAP activity with the Phospha-Light kit, using CSPD as a luminogenic substrate and light production was measured in a Topcount luminometer. Comparison with untreated cells shows a 4-fold increase in SEAP activity when the cells were treated with IFNβ or IFNα. There was no induction of SEAP by EPO in the cells transfected with the EPO-R/IFNaR1 chimer alone. However, a 3 to 4-fold induction of SEAP activity by EPO was observed in those cells transfected with the EPO-R/IFNaR1 + EPO-R/IFNaR2-2 constructs or with the EPO/IFNaR2-2 construct alone (figure 2)indicating that at least EPO-R/IFNaR2-2 can be activated by EPO and transmits a signal resulting in 6-16 promoter activation.

III. ACTIVATION OF THE CHIMERIC RECEPTORS UPON ENDOGENOUSLY PRODUCED LIGAND

III.1. Construction of the vectors pEFBos-hIL-5syn and pMET7-hIL-5syn for constitutive eukaryotic expression of IL-5.

The gene for hIL-5syn was isolated from the pGEM1-hIL-5syn vector (Tavernier *et al.* 1989) by Sal I digestion and agarose gelelectrophoresis. The fragment was cloned into the Sal I opened pEFBOS (gift from Nagata,S., Osaha Bioscience Institute, Japan) or pMET7MCS vectors . The latter vector was constructed by insertion of a multicloning site (Sal I-Bgl II-EcoRV-BstEII-Age I-Xho I-Xba I)) into the vector pMET7-Lrlo (gift from L. Tartaglia, Millenium, Cambridge), using a synthetic DNA fragment, formed by hybridization of the oligonucleotides MBU-O-187 and MBU-O-188 (table 1). As a result, the hIL-5syn gene was cloned downstream the promoter for human elongation factor 1a (HEF1a, Mizushima *et al.,* 1990) or downstram

the hybrid SRα promoter (Takebe *et al.* 1988) and the plasmid were named pEFBos-hIL-5syn and pMET7-hIL-5syn, respectively.

III.2. Chimeric receptors allow survival selection upon endogeneously produced ligand.

The plasmid pEFBOS-hIL-5synor the pUC18 vector (mock) were used for transfection of 2ftGH cells that stabile expressed the IL-5Rα/IFNaR2-2 + β c/IFNaR1 chimeras (2ftGH clone C cells). Transfection was performed overnight according to the Ca-phosphate method. The precipitates were made up in 1 ml and left on the cells overnight (5 x 10^5 cells / transfection / petridish). The next day, cells were washed twice with Dulbecco's PBS. Two days later, cells were incubated on HAT medium alone, after which cell survival was visually followed using an inverted microscope. Three days later, a clear difference in cell confluency between pEFBOS-hIL-5syn and mock transfected cells was visible. The cells were trypsinised and a limited dilution was set up in a 96-well microtiterplate from the cells, transfected with pEFBOS-hIL-5syn. Six colonies, arising from a single cell and surviving in HAT medium without IL-5 supplementation could be isolated, indicating that these cells produced IL-5 and stimulated the chimeric receptor in an autocrinic fashion.

III.3. Determination of the minimum amount of pEFBOS-hIL-5syn DNA required for generation of an IL-5 autocrinic loop

A 1 :10 dilution series of pEFBOS-hIL-5syn DNA in irrelevant DNA (pcDNA.3) was set up : 1.5 (1/10), 0.15 (1/100), 0.015 (1/1000) and 0.0015 (1/10000) µg of pEFBOS-hIL-5syn DNA were added to 15 µg pcDNA3 DNA and transfected in the IL-5Rα/IFNaR2-2 + βc/IFNaR1 clone C cells. Positive and negative controls were 15 µg of pEFBOS-hIL-5syn and 15 µg of pcDNA3, respectively. Transfection was according to the Ca-phosphate procedure. The precipitates were made up in 1 ml and left on the cells overnight (5 x 10^5 cells / transfection / petridish). Following washing (2 x with Dulbecco's PBS),

DMEM medium was added for 24 hours after which it was changed to HAT medium. Cells were visually followed using an inverted microscope and 15 days after transfection, photographs of representative regions in every petri dish were taken. All of the petri dishes containing cells transfected with one of the pEFBOS-hIL-5syn dilutions, showed a marked increase in cell number as compared to the negative control (figure 3). Hence, transfection of as little as 1.5 ng pEFBOS-hIL-5syn is sufficient to generate an autocrinic loop that can sustain cell survival.

III.4. Determination of the minimum amount of pMET7-hIL-5syn DNA required for generation of an IL-5 autocrinic loop.

A dilution series of pMET7-hIL-5syn DNA in irrelevant DNA (pCDNA3) was set up : 4 ng ($1/10^4$), 400 pg ($1/10^5$), and 40 pg ($1/10^6$) of pEFBOS-hIL-5syn DNA were added to 40 µg pCDNA3 DNA and transfected in the clone E cells (stabile transfected with pSV-SPORT-IL-5Rα/IFNaR2-2 + pSV-SPORT-β c/IFNaR1). As a negative control, 40 µg of pCDNA3 alone was used. Every precipitate was prepared in 1 ml according to the Ca-phosphate procedure, from which 165 µl was brought onto 10^5 cells in the well of a 6-well microtiterplate. The precipitate was left on the cells overnight after which cells were washed twice with Dulbecco's PBSand further grown in DMEM medium. After 24 hours, medium samples were taken from each well and SEAP activity was measured using the Phospha-Light assay. Luminescence was measured in a Topcount luminometer (figure 2). Transfection of 2ftGH cells with 400 pg pMET7-hIL-5syn ($1/10^5$ dilution) still resulted in the formation of an autocrinic loop as shown by the production of SEAP in the medium (figure 4).

Short description of the Figures

Figure 1: Transient co-transfection of pSV-SPORT-IL-5Ra/IFNaR2-2, pSV-SPORT-bc/IFNaR1 and p6-16SEAP in 2ftGH cells and analysis of induction of SEAP activity. 24 hours after transfection, cells were left unstimulated or were stimulated with IFNb (positive control) or IL-5 (1 and 2 ng/ml). Samples from the medium were taken 24 hours after stimulation and SEAP activity was

measured using CSPD as a luminogenic substrate. The amount of light produced was determined in a Topcount luminometer.

Figure 2: Transient transfection of pSV-SPORT-EPO-R/IFNaR1 + pSV-SPORT-EPO-R/IFNaR2-2, pSV-SPORT-EPO-R/IFNaR2-2, pSV-SPORT-EPO-R/IFNaR2-2 or pUC18 (mock) in 2ftGH-6-16SEAP cells (clone 2). 24 hours after transfection, cells were left unstimulated or were stimulated with IFNa (positive control) or EPO. Samples from the medium were taken 24 hours after stimulation and SEAP activity was measured using CSPD as luminogenic substrate. The amount of light was determined in a Topcount luminometer.

Figure 3: Survival of cells IL-5Ra/IFNaR2-2 + bc/IFNaR1 clone C cells, transfected with dilutions of the vector pEFBOS-hIL-5syn in irrelevant DNA. Formation of an autocrinic loop results in survival of the cells in HAT medium. Fifteen days after transfection, photographs of representative regions in each petridish were taken.

Figure 4: Induction of SEAP activity in IL-5Ra/IFNaR2-2 + bc/IFNaR1 clone E, transfected with dilutions of the vector pMET7-hIL-5syn in irrelevant DNA and co-transfected with the p6-16 plasmid. Formation of an autocrinic loop results in activation of the 6-16 promoter followed by secretion of SEAP. Samples from the medium were taken 24 hours after transfection and SEAP activity was measured using CSPD as luminogenic substrate. The amount of light produced was determined in a Topcount luminometer.

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Claims

1. An eukaryotic cell comprising 1) a chimeric receptor 2) a recombinant gene, encoding a compound of which the expression creates an autocrinic or anti-autocrinic loop 3) a reporter system that is activated or inactivated upon the creation of said autocrinic or anti-autocrinic loop.
2. An eukaryotic cell according to claim 1 in which the cell is an insect cell, a plant cell or a mammalian cell
3. An eukaryotic cell according to claim 1 or 2 in which the chimeric receptor is a multimeric receptor.
4. An eukaryotic cell according to claim 1 - 3 in which said recombinant gene is placed after a constitutive promoter.
5. An eukaryotic cell according to claim 1 - 4 in which said reporter system is activated as a result of the binding of a ligand to said chimeric receptor.
6. An eukaryotic cell according to any of the preceeding claims in which the cytoplasmic part of the chimeric receptor is the cytoplasmic part of the interferon receptor
7. An eukaryotic cell according to any of the preceeding claims in which the reporter system is *E. coli* xanthin-guanin phosphoribosyl transferase.
8. An eukaryotic cell according to claim 6 in which said reporter system is placed under control of the 6-16 promoter
9. An eukaryotic cell according to claim 4 in which said recombinant gene is placed after the SRa or the HEF1a promoter
10. An eukaryotic cell according to any of the preceeding claims in which the cell is a 2ftGH cell.
11. The use of an eukaryotic cell according to any of the preceeding claims for screening of orphan receptors and/or unknown ligands
12. The use of an eukaryotic cell according to claim 1-10 to screen compounds that interfere with the binding of a ligand with the extracellular part of said chimeric receptor and/or with the signalling pathway of the cytoplasmic part of said chimeric receptor.
13. A method for screening orphan receptors and/or unknown ligands comprising a) transformation of an eukaryotic host cell with a gene

encoding a chimeric receptor b) transformation of said host cell with a gene encoding a reporter system inducible by the binding of a ligand to said chimeric receptor c) transformation of said host cell with a gene encoding for a ligand of said chimeric receptor d) selection for cells in which the reporter system is activated or inactivated.

14. Orphan receptors and/or unknown ligands, obtainable by the method of claim 13.

15. A method for screening compounds that interfere with the binding of a ligand to a receptor and/or with the signalling pathway of a receptor, comprising a) transformation of an eukaryotic host cell with a gene encoding a chimeric receptor b) transformation of said host cell with a reporter system inducible by the binding of a ligand to said chimeric receptor c) transformation of said host cell with a gene encoding an inhibitor of the binding of said ligand to said chimeric receptor d) transformation said host cell with a gene encoding a ligand for said chimeric receptor and/or supplying said ligand to the host cell e) selection for cells in which the reporter system is activated or inactivated

16. A kit, comprising an eukaryotic host cell and one or more transformation vectors, which upon transfection of said cell with said vector or vectors results in an eukaryotic cell according to claim 1-10.

Abstract

The present invention relates to a method for screening compounds for their ability to bind a receptor and/or the screening of compounds that antagonise the binding of a ligand to a receptor.

It is the aim of the present invention to provide an easy and powerful screening method in eukaryotic cells, such as insect cells, plant cells or mammalian cells, with the exclusion of yeast cells, for ligands of orphan receptors, preferentially of the multimerizing receptor type, for unknown ligands of known receptors, preferentially multimerizing receptors and for the genes encoding these ligands.

Figure 1

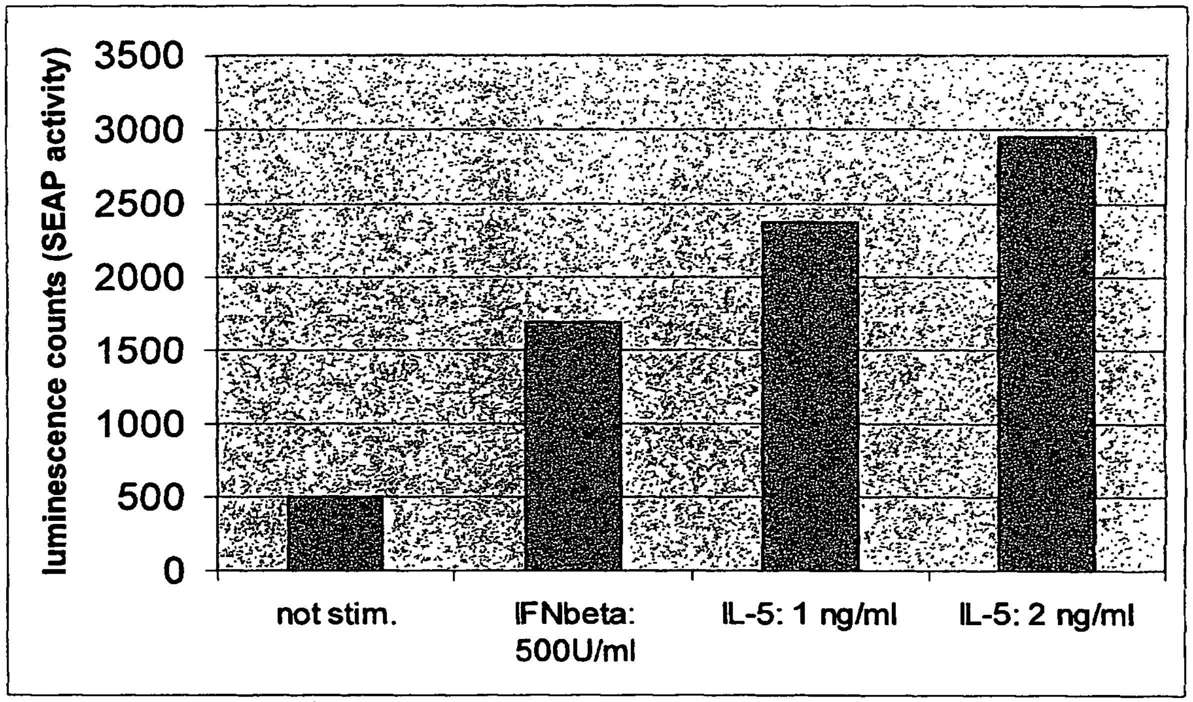

luminescence counts (SEAP activity)
3500
3000
2500
2000
1500
1000
500
0
not stim.
IFNbeta: 500U/ml
IL-5: 1 ng/ml
IL-5: 2 ng/ml

Figure 2

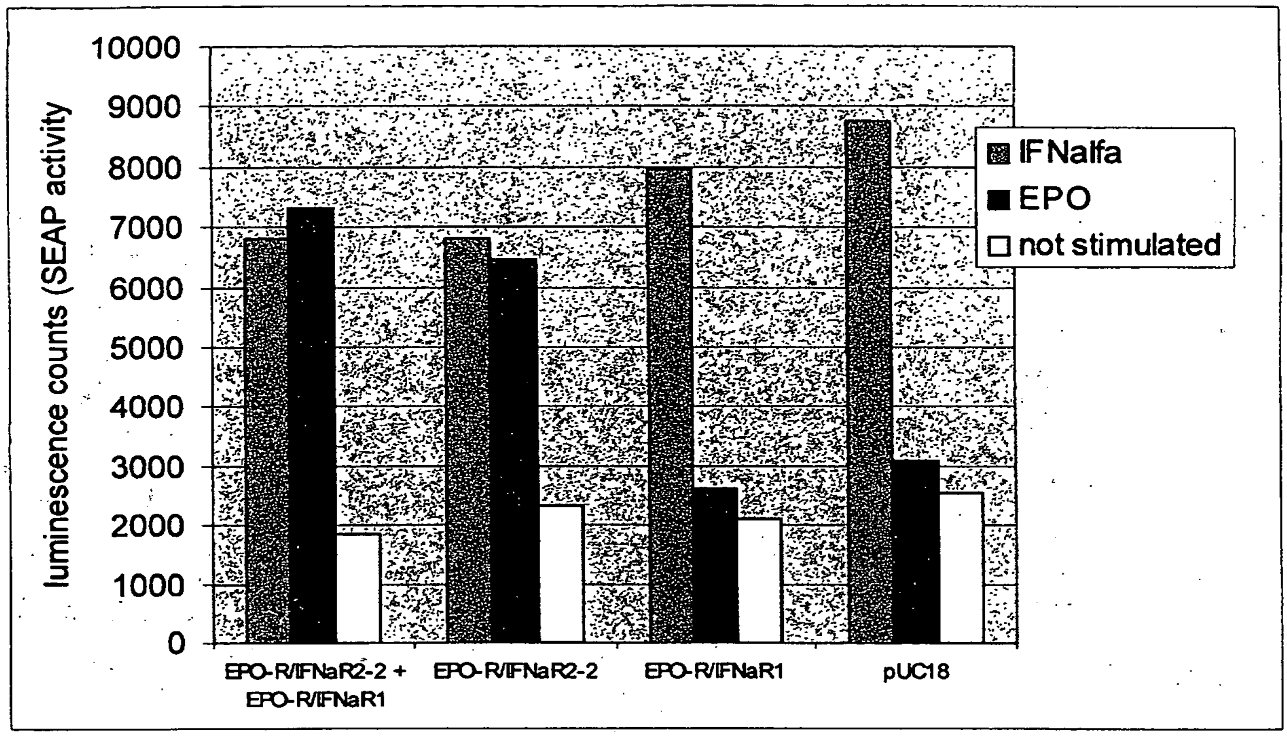

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
luminescence counts (SEAP activity
IFNalfa
EPO
not stimulated
EPO-R/IFNaR2-2 + EPO-R/IFNaR1
EPO-R/IFNaR2-2
EPO-R/IFNaR1
pUC18

Figure 3

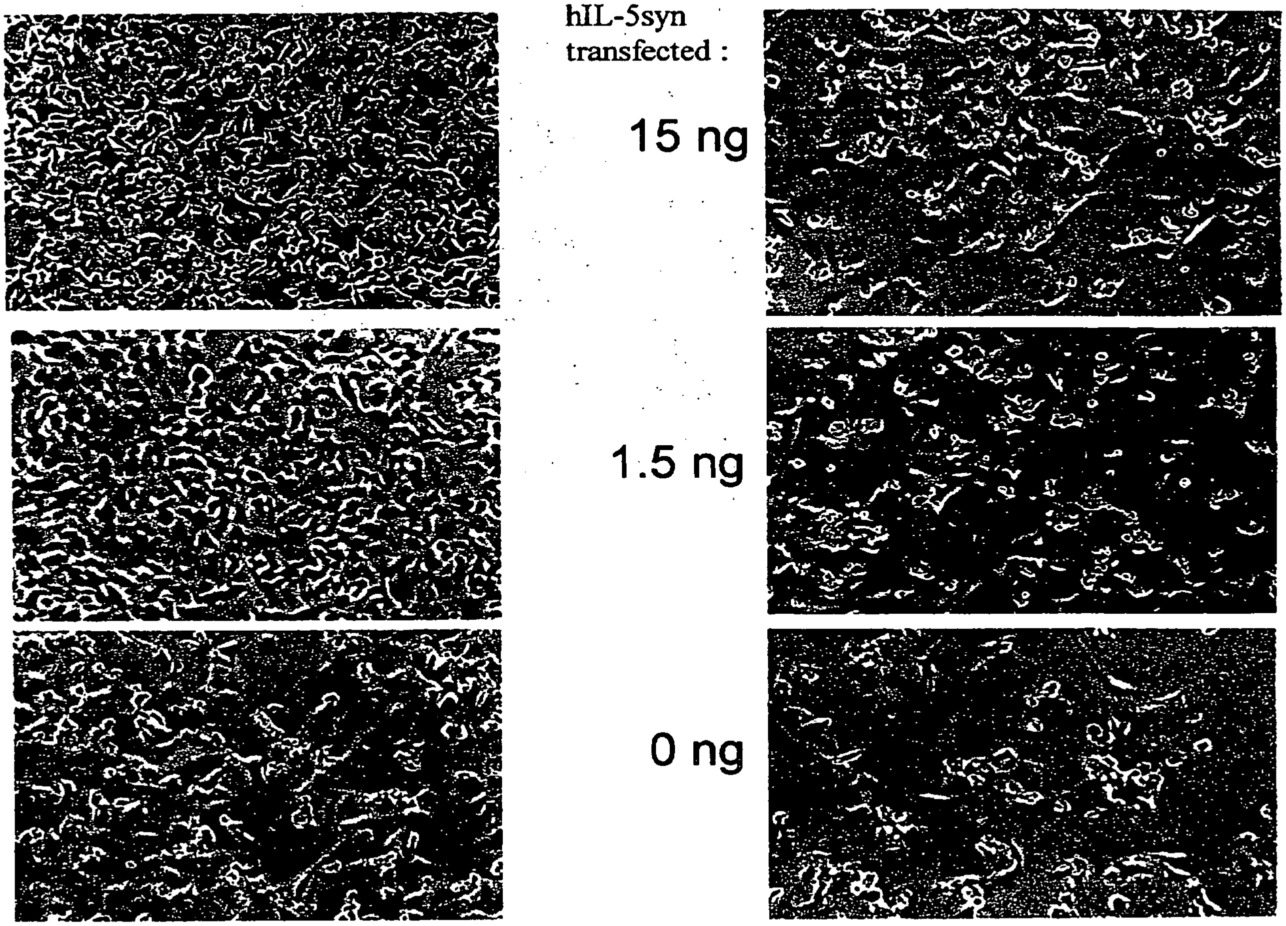
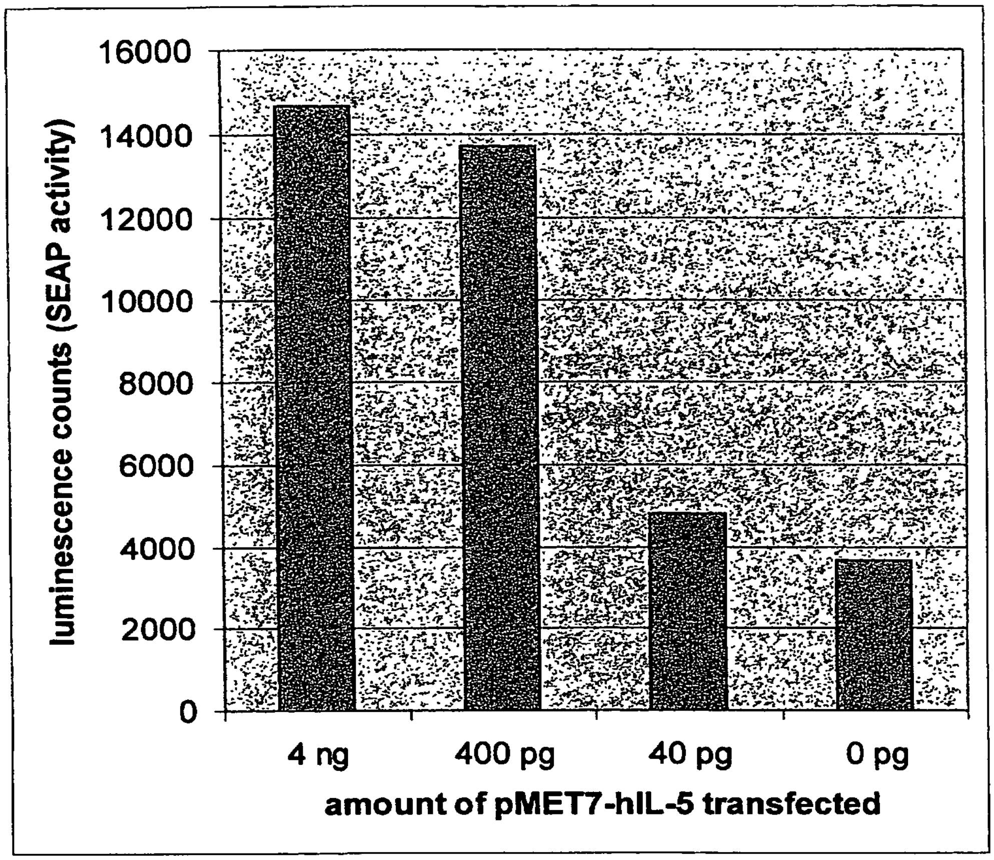

Amount of pEFBOS-hIL-5syn transfected :
15 µg
1.5 µg
150 ng
Amount of pEFBOS-hIL-5syn transfected :
15 ng
1.5 ng
0 ng

Figure 4


16000
14000
12000
10000
8000
6000
4000
2000
0
luminescence counts (SEAP activity)
4 ng
400 pg
40 pg
0 pg
amount of pMET7-hIL-5 transfected